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Bcl-2 Inhibitors Sensitize Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand-Induced Apoptosis by Uncoupling of Mitochondrial Respiration in Human Leukemic CEM Cells

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ABSTRACT

Previous studies have shown that the lymphoblastic leukemia CEM cell line is resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis because of a low expression of caspase-8. Bcl-2 inhibitors, BH3I-2′ and HA14-1, are small cell-permeable nonpeptide compounds, are able to induce apoptosis by mediating cytochrome *c* release, and also lead to dissipation of the mitochondrial membrane potential ($\Delta\Psi m$). This study aimed to use the Bcl-2 inhibitors to sensitize CEM cells to TRAIL-induced apoptosis by switching on the mitochondrial apoptotic pathway. We found that a low dose of BH3I-2′ or HA14-1, which did not induce cytochrome *c* release, greatly sensitized CEM cells to TRAIL-induced apoptosis. In a similar manner to the classical uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), both BH3I-2′ and HA14-1 induced a reduction in $\Delta\Psi m$, a generation of reactive oxygen species (ROS), an increased mitochondrial respiration, and a decreased ATP synthesis. This uncoupling function of the Bcl-2 inhibitors was responsible for the synergy with TRAIL-induced apoptosis. CCCP *per se* did not induce apoptosis but again sensitized CEM cells to TRAIL-induced apoptosis by uncoupling mitochondrial respiration. The uncoupling effect facilitated TRAIL-induced Bax conformational change and cytochrome *c* release from mitochondria. Inhibition of caspases failed to block TRAIL-mediated cell death when mitochondrial respiration was uncoupled. We observed that BH3I-2′, HA14-1, or CCCP can overcome resistance to TRAIL-induced apoptosis in TRAIL-resistant cell lines, such as CEM, HL-60, and U937. Our results suggest that the uncoupling of mitochondrial respiration can sensitize leukemic cells to TRAIL-induced apoptosis. However, caspase activation *per se* does not represent an irreversible point of commitment to TRAIL-induced cell death when mitochondrial respiration is uncoupled.

INTRODUCTION

Apoptosis initiated by TRAIL is largely dependent on the cell-extrinsic signaling pathway, which involves death receptor engagement, the death-inducing signaling complex formation, proteolytic activation of the apical caspases, caspase-8 and -10, and consequently, activation of effector caspases such as caspase-3, -6, and -7 (1, 2). In certain types of cells, effector caspase activation requires amplification of death-inducing signaling complex signals by engagement of the cell-intrinsic pathway. A critical step in the cell-intrinsic pathway is the activation of Bax, leading to dissipation of the mitochondrial transmembrane potential ($\Delta\Psi m$) and cytochrome *c* release into the cytosol. This facilitates assembly of the Apaf-1 apoptosome with recruitment and activation of caspase-9 and subsequently the effector caspases (3). Multidomain proapoptotic members of the Bcl-2 family, such as Bax and Bak, are counterbalanced by the antiapoptotic family members Bcl-2 or Bcl-XL (4). BH3-only proteins, such as Bid, interact with proapoptotic Bcl-2 family members to augment their activity. Once cleaved by caspase-8 during treatment with TRAIL, Bid translocates to the mitochondria and activates Bax, thus providing a mechanism for cross-talk between the extrinsic and intrinsic apoptotic pathways (5, 6).

The requirement for Bax activation in TRAIL-induced apoptosis is cell type dependent (7–10). Early events triggered by TRAIL, such as death-inducing signaling complex formation, caspase-8 activation, and Bid cleavage were not dependent on Bax; however, mitochondrial depolarization, cytochrome *c* release, and activation of caspase-9 were prevented in Bax-deficient cells (9, 11). Thus, in these cells, the intrinsic pathway was required for TRAIL-mediated apoptosis, with Bax being essential for induction of the mitochondrial events.

BH3I-2′ and HA14-1 are small nonpeptidic organic compounds that interact with the surface pocket of Bcl-2 and can be used as cell-permeable agents to affect Bcl-2-regulated apoptotic pathways and are called inhibitors of Bcl-2 or BH3-mimetic compounds (12, 13). These small compounds not only induce cytochrome *c* release from mitochondria but also dissipate $\Delta\Psi m$ (12–14).

The mitochondria of healthy cells maintain an electrochemical gradient across the mitochondrial inner membrane (MIM) that is created by pumping protons from the matrix to the inter-membrane space of these organelles in conjugation with electron transport through the respiratory chain. The proton gradient and membrane potential are the proton-motive force that is used to drive ATP synthesis. Coupling of electron transport through the respiratory chain and ATP generation can be disrupted by some acidic aromatic substances such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol. These so-called uncouplers of oxidative phosphorylation carry protons across the inner mitochondrial membrane. This specific attack of oxidative phosphorylation leads to a reduction of $\Delta\Psi m$, to the cessation of ATP generation in the mitochondrion, and to the collapse of the pH gradient by shuttling protons back across the membrane (15).

Alteration in mitochondrial function can change the sensitivity of tumor cells to apoptosis mediated by death receptors. Increase in mitochondrial respiration sensitizes leukemic cells to tumor necrosis factor-mediated apoptosis (16). Depletion in mitochondrial DNA renders tumor cells resistant to apoptosis induced by TRAIL (17). The uncoupler CCCP can enhance the Fas death signal, although CCCP alone does not have an apoptotic effect (18). However, the precise mechanism by which the mitochondrial function contributes to death receptor-mediated apoptosis is still unclear.

In this study, we used BH3I-2′ or HA14-1 as a sensitizer for overcoming the resistance of leukemic cells to TRAIL-induced apoptosis. It was found that both BH3I-2′ and HA14-1 showed an uncoupling effect on the oxidative phosphorylation when they were used at the concentrations that could not induce cytochrome *c* release and apoptosis. CCCP, which does not induce apoptosis, also showed a large synergistic effect on TRAIL-induced apoptosis in leukemic cells. Our data showed that the synergistic effect of uncoupling agents on TRAIL-induced apoptosis is via the intrinsic apoptotic pathway

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Note: J-H. Hao and M. Yu contributed equally to this work.

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(*i.e.*, to enhance Bax conformational change, cytochrome *c* release, and caspase-3 activation).

MATERIALS AND METHODS

Materials. TRAIL was obtained from Affiniti-Biomol (Exeter, United Kingdom). BH3I-2′, dihydroethidium, ATP assay kit, and Z-Asp-Glu-Val-Asp-AFC (Z-DEVD-AFC) were purchased from Novabiochem-Calbiochem (Nottingham, United Kingdom). HA14-1 was obtained from Qbiogene-Alexis Ltd. (Nottingham, United Kingdom). Tetramethylrhodamine methylester, 2′,7′-dichlorodihydrofluorescein diacetate, Mito-Tracker red CMXRos, and 5-(and-6)-chloromethyl SNARF-1 acetate were obtained from Molecular Probes (Eugene, OR). The caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD.fmk), was obtained from Promega (Southampton, United Kingdom). Mouse anticytochrome *c* antibody (6H2.B4) and monoclonal mouse anti-Bax (clone 6A7) were obtained from BD PharMingen (San Diego, CA). Monoclonal mouse anti-Bax antibody (clone YTH-2D2) was from R&D Systems (Oxon, United Kingdom). Dynabeads M-450 rat antimouse IgG1 was purchased from Dynal Biotech Ltd. (Wirral, United Kingdom). CCCP, gum agar, nigrericin, propidium iodide (PI), RNase A, monoclonal anti-β-actin antibody, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all chemicals were purchased from Sigma (Dorset, United Kingdom).

Cell Culture and the Treatment of Cells. The human T-lymphoblastic leukemia CEM, the promyelocytic leukemia HL-60, the promonocytic leukemia U937, and the chronic erythroleukemia K562 cell lines were used in this study, and cell culture was performed as described previously (16). For the single treatment of cells, 10^6 cells/ml were treated with 500 ng/ml TRAIL, 30 μM BH3I-2′, 10 μM HA14-1, or 10 μM CCCP for up to 24 h. For the cotreatment, cells were pretreated with 30 μM BH3I-2′, 10 μM HA14-1, or 10 μM CCCP for 1 h and then treated together with 500 ng/ml TRAIL for up to 24 h.

Apoptosis Assay by Flow Cytometry. DNA content was measured by flow cytometry. Cells were permeabilized with 70% ethanol and stained with 100 μg/ml PI. PI fluorescence of nuclei was measured by a FACScan flow cytometer (Becton Dickinson, Cambridge, United Kingdom). Data analysis was carried out on cells gated on an FL2-Area channel *versus* FL2-Width channel display to exclude cell debris and clumped cells. DNA content distribution (PI fluorescence) was analyzed on the FL2-Area histogram, and cells with a DNA content less than G_0/G_1 (hypodiploid) were defined as apoptotic cells (7).

Soft Agar Assay for Colony Formation. Two percent of gum agar was melted in a microwave and cooled to 50–60°C in a water bath. Eagle's Minimum Essential Medium (EMEM) and 20% FCS were prewarmed to 40°C in water bath. Agar was mixed with medium and FCS to give 0.5% agar and 10% FCS. Two ml of 0.5% agar were added to each 35-mm Petri dish and allowed to set. The top agar was prepared with 2% agar, EMEM, and FCS to give 0.3% agar and 10% FCS. Agar (1.8 ml of 0.3%) was mixed with 0.2 ml of CEM cell suspension (containing 2×10^4 cells) by vortexing the contents vigorously until the cells were evenly suspended. The cell-containing mixture was plated in a 2-ml volume on the top agar. The dish was overlaid with 1 ml of EMEM containing supplements and with or without 30 μM BH3I-2′ or 10 μM HA14-1. Cells were incubated for 1 week at 37°C in 5% CO_2 before counting colonies (19).

Detection of ΔΨm and ROS Generation by Flow Cytometry. For measuring ΔΨm, CEM cells were stained with 20 nM tetramethylrhodamine methylester for 30 min at 37°C. The fluorescent intensities were measured in the FL3-H channel with a FACScan flow cytometer. The intracellular accumulation of ROS was determined using the fluorescent probe dihydroethidium to measure O_2^- at FL3-H and 2′,7′-dichlorodihydrofluorescein diacetate for H_2O_2 at FL1-H. After the treatment, cells (10^5/ml) were incubated with 40 μM dihydroethidium and 5 μM 2′,7′-dichlorodihydrofluorescein diacetate for 15 min at 37°C. ROS generation was then assessed by a FACScan flow cytometer at both FL1-H and FL3-H channels.

Measurement of Caspase-3 Activation. The caspase-3 activity was measured by a fluorogenic method using Z-DEVD-AFC as a substrate. The fluorescence of AFC at 400/505 nm was measured with a TD-700 fluorometer (Turner Design, Sunnyvale, CA). Caspase activity was defined as micromolar AFC release per hour per milligram protein (μM/h/mg protein; Ref. 19).

Measurement of Mitochondrial Respiration. CEM cells were suspended in the respiratory medium [250 mM sucrose, 20 mM HEPES, 10 mM $MgCl_2$, 5 mm KH_2PO4, 0.1% BSA, 1 mM ADP (pH 7.4)]. Oxygen consumption of leukemic cells was measured with the rank oxygen electrode (Rank Brothers, Cambridge, United Kingdom) in a thermojacked sample chamber stirred with a magnetic flea. One ml of cell suspension containing 10^7 cells was added to the 1-ml sample chamber (16). The respiratory rate was monitored for 3 min and then 10 μM CCCP, 30 μM BH3I-2′, or 10 μM HA14-1 was added to the suspension and recorded for another 3 min. Oxygen consumption was calibrated with air-saturated respiratory medium assuming 390 ng atoms O_2/ml. The respiratory rate was expressed as nanogram atoms of O_2 per minute per 10^7 cells.

ATP Measurement. Intracellular ATP content was determined using a bioluminescence assay kit according to the Calbiochem manufacturer's suggestions. Cells (1×10^6) were treated with drugs as described. Cells (10^4) were taken out at 4 h. After washing once with Hanks buffer, cells were lysed with releasing reagent. Immediately after solubilization, the intracellular ATP content was measured with the luciferin-luciferase assay in a TD-20/20 Luminometer (Turner Design, Sunnyvale, CA). ATP content was calculated using an ATP standard curve and expressed as nanomolar ATP released from 10^7 cells (nM/10^7 cells; Ref. 20).

Determination of Intracellular pH Value. CEM cells were suspended in HEPES-buffered medium (without serum) and incubated with 5 μM SNARF-1 acetate for 30 min at 37°C. After centrifuge, cells were resuspended in fresh medium. A pH calibration curve was generated by preloading cells with 5 μM SNARF-1 acetate, followed by incubation for 30 min in different pH buffers (from 6.5 to 8) in the presence of permeabilizing agent nigrericin (10 μM) in a high-K^+ HEPES buffer (135 mM KH_2PO_4/K_2HPO_4, 20 mM HEPES, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose). Fluorescence intensities were measured in both FL2-H and FL3-H channels. The ratio of FL3-H/FL2-H fluorescence intensity was proportional to the intracellular pH value (21).

Bax Conformational Change. Cells were washed with PBS and lysed with Chaps buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 1% Chaps, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 25 μg/ml leupeptin, and 25 μg/ml pepstain]. One μg of anti-Bax (6A7) monoclonal antibody was preincubated with 20 μl of Dynabeads (M-450 rat antimouse IgG1) at 4°C on the rotor for 3 h. The cell lysates were normalized for protein content, and 1000 μg of total protein in 300 μl Chaps lysis buffer were then added to the immuno-precipitation tube containing Bax antibody (6A7)-loaded Dynabeads and incubated at 4°C on the rotor overnight. After rinsing four times with Chaps buffer, beads were collected with a Dynal Magnetic Particle Concentrator (Dynal). Conformationally changed Bax protein was eluted with 25 μl of sample buffer for Western blotting by the monoclonal anti-Bax antibody, clone 2D2 (22).

Immunofluorescence Analysis of Cytochrome *c* Release. To colocalize cytochrome *c* in mitochondria, intact cells were first labeled with the mitochondrion-specific dye, MitoTracker red CMXRos. Cells in culture medium were incubated with MitoTracker (100 nM) at 37°C for 30 min. After washing, cells were fixed/permeabilized on slides. Cells were incubated with the anti-cytochrome *c* antibody 6H2.B4 (1:400 dilution) for 2 h and then incubated with FITC-conjugated antimouse secondary antibody (Sigma) at a 1:20 dilution. Slides were air dried at 4°C in the dark and stained with 4′,6-diamidino-2-phenylindole before being viewed under a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Oberkochen, Germany; Ref. 23).

Measurement of Cell Membrane Integrity by PI Dye Exclusion. After treatment, cells were stained with 10 μg/ml PI for 5 min at room temperature. The PI fluorescence was measured by flow cytometry in the FL3-H channel. Intact cells were PI negative, and PI-positive cells were defined as necrotic cells with a broken cell membrane.

Measurement of Cytotoxicity by the MTT Test. MTT can be cleaved by mitochondrial enzymes, mainly by succinate dehydrogenase, to form a dark blue crystalline product, formazan. Reduced formation of formazan is caused by decreased mitochondrial dehydrogenase activity, inhibited cell proliferation, or cell death (14). Cells (10^5/ml) were treated for 24 h; MTT dye was added to each well to reach the final concentration of 50 μg/ml and allowed to incubate for 4 h at 37°C. Plates were centrifuged and media replaced with 150 μl of isopropyl alcohol containing 0.4 N HCl to solubilize the insoluble formazan complex. Absorbance at 540 nm was determined with an ELISA plate reader.

RESULTS

Bcl-2 Inhibitor, BH3I-2′ or HA14-1, Sensitizes Leukemic CEM Cells to TRAIL-Induced Apoptosis. Human leukemic CEM cells are relevantly resistant to TRAIL-induced apoptosis because of a low level of caspase-8, induction of Bcl-2, and degradation of Bax protein (24). We aimed to study whether switching on the mitochondrial apoptotic pathway could sensitize CEM cells to TRAIL-induced apoptosis. Measurement of apoptotic cells was performed by fixing cells, staining with PI, and analyzing by flow cytometry after treatment with 500 ng/ml of TRAIL. Apoptotic cells were defined as DNA content less than G_0/G_1 (Fig. 1*A*). The cell- permeable inhibitors for Bcl-2, BH3I-2′, and HA14-1 were used to test the synergistic effect on TRAIL-induced apoptosis. BH3I-2′ did not significantly induce apoptosis at concentrations <50 μM within 4 h. The concentration of BH3I-2′, which showed a significant synergistic effect on TRAIL-induced apoptosis (*t* test, $P < 0.001$), started at 30 μM (Fig. 1*Ba*). Therefore, 30 μM BH3I-2′ were used to study the synergistic effect on TRAIL kinetically within 6 h, and it was shown (Fig. 1*Bb*) that BH3I-2′ significantly increased the sensitivity of CEM cells to TRAIL-induced apoptosis compared with cells treated with TRAIL

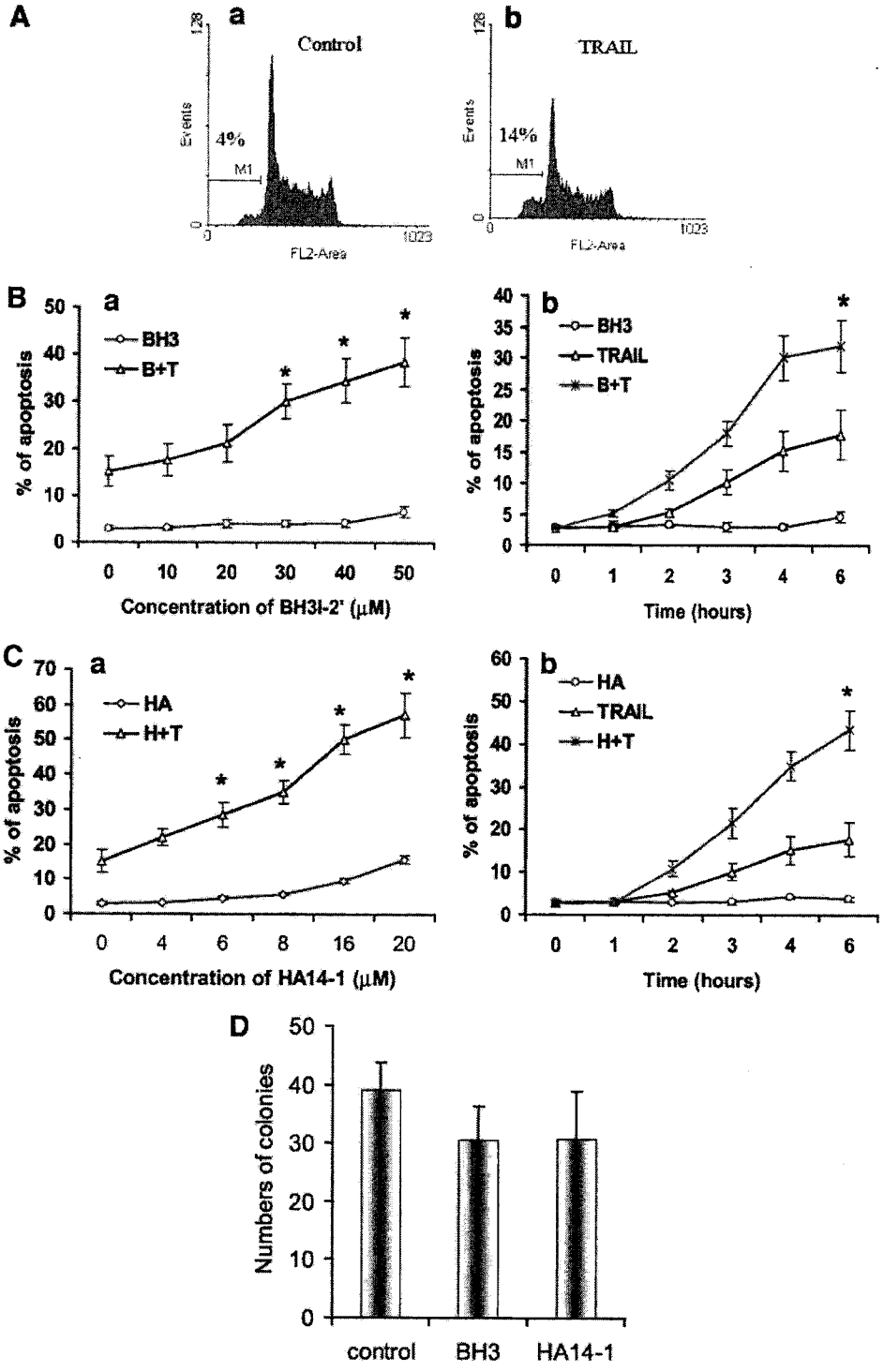


Fig. 1. Synergistic effect of Bcl-2 inhibitors on TRAIL-induced killing. *A*, flow cytometry assay for apoptosis. Histogram of DNA content was obtained from FL2-Area *verses* cell numbers. Control CEM cells (*a*) and CEM cells (*b*) were treated with TRAIL. Cells with DNA content less than G_0/G_1 (M1) are apoptotic cells. *B*, synergistic effect of BH3I-2′ on TRAIL-induced apoptosis. Dose-dependent course (*a*). CEM cells were pretreated with different doses of BH3I-2′ for 1 h and then incubated with (B+T) or without (BH3) 500 ng/ml TRAIL for 4 h. Time-dependent course (*b*). CEM cells were pretreated with 30 μM BH3I-2′ for 1 h and then treated with 500 ng/ml TRAIL for 6 h. Cells treated with TRAIL alone were used as control. Cells were collected at each indicated time point. *C*, synergistic effect of HA14-1 on TRAIL-induced apoptosis. Dose-dependent course (*a*). CEM cells were pretreated with different doses of HA14-1 for 1 h and then incubated with (H+T) or without (HA) 500 ng/ml TRAIL for 4 h. Time-dependent course (*b*). CEM cells were pretreated with 10 μM HA14-1 for 1 h and then treated with 500 ng/ml TRAIL for 6 h. Significantly increased sensitivity ($*P < 0.001$) was statistically compared in cells that were treated with TRAIL and BH3I-2′ or HA14-1 with those treated with TRAIL alone. *D*, colony formation assay. CEM cells in soft agar were treated with 30 μM BH3I-2′ or 10 μM HA14-1 for 1 week. Cell numbers were counted in each grid. Data shown were mean ± SD from three independent experiments. *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand.

alone (ANOVA, $P < 0.001$). The concentration of HA14-1, which showed a significant synergy (t test, $P < 0.001$) with TRAIL, started at about 6 μM (Fig. 1*Ca*). Ten μM HA14-1 were used to assess the sensitization effect on TRAIL kinetically (Fig. 1*Cb*), and again it showed a significant synergistic effect on TRAIL-induced apoptosis (ANOVA, $P < 0.0001$). The colony formation assay in soft agar was used to examine whether 30 μM BH3I-2′ or 10 μM HA14-1 alone have an inhibitory effect on cell proliferation. It was observed that 30 μM BH3I-2′ or 10 μM HA14-1 only slightly but not significantly inhibited colony formation (Fig. 1*D*). These results imply that Bcl-2 inhibitors can sensitize TRAIL-induced apoptosis, and this sensitization is not a simple additive effect.

Bcl-2 Inhibitor Showed Similarities to the Classical Uncoupler CCCP. Previously we observed that BH3I-2′ induces both cytochrome *c* release from mitochondria and reduction in $\Delta\Psi$m at the concentration of 70 μM, and these can be inhibited by Bcl-2/Bcl-XL (14). In this study, we used 30 μM BH3I-2′ or 10 μM HA14-1, which could not induce cytochrome *c* release nor apoptosis within 12 h (data not shown). We found that both BH3I-2′ (30 μM) and HA14-1 (10 μM) induced a collapse in $\Delta\Psi$m in the CEM cell line after treatment for 2 h (Fig. 2*A*), as measured by flow cytometry when cells were stained with tetramethylrhodamine methylester dye. Generation of ROS, O_2^-, and H_2O_2, were examined by flow cytometry. Both BH3I-2′ and HA14-1 initiated O_2^- and H_2O_2 generation simultaneously, shown in the shift of the cell population from the *bottom-left quarter* (control) to the *top-right quarter* (O_2^-, red; H_2O_2, green; Fig. 2*B*). CCCP was initially used as a positive control for the reduction of $\Delta\Psi$m and the generation of ROS. Results indicated that Bcl-2 inhibitors showed some similar effects to the uncoupler CCCP on the MIM (*i.e.*, the reduction of $\Delta\Psi$m and the generation of ROS). It had been shown previously that the CEM cell line is relatively resistant to TRAIL-induced reduction in $\Delta\Psi$m (24). When combined with BH3I-2′, HA14-1, or CCCP, TRAIL-mediated dissipation in $\Delta\Psi$m was greatly enhanced (Fig. 2*A*). TRAIL alone did not induce production of ROS, and it did not show synergistic effect on BH3I-2′, HA14-1, or CCCP-induced generation of ROS (Fig. 2*B*).

We therefore investigated whether the uncoupler CCCP could sensitize CEM cells to TRAIL-induced apoptosis. The proapoptotic effect of CCCP on TRAIL was tested at both 4 and 24 h. CCCP did not induce apoptosis in CEM cells when used alone but showed a significant synergistic effect when it was combined with TRAIL. The percentages of apoptotic cells increased to 60–70% when combined with CCCP compared with about 10–20% apoptotic cell death when CEM cells were treated with TRAIL alone at 24 h (Fig. 2*C*).

The synergistic effect of Bcl-2 inhibitors and CCCP on TRAIL-induced apoptosis was also examined on other leukemic cell lines. Both HL-60 (Fig. 3*A*) and U937 (Fig. 3*B*) cell lines were relatively resistant to TRAIL-induced apoptosis. However, TRAIL-induced apoptosis was significantly increased in both HL-60 and U937 cell lines when cells were pretreated with BH3I-2′, HA14-1, or CCCP (Fig. 3, *A* and *B*). The K562 cell line is sensitive to TRAIL-induced apoptosis, as reported previously (19, 24). Neither Bcl-2 inhibitors nor CCCP further sensitized K562 cells to TRAIL-induced apoptosis (Fig. 3*C*).

To test whether these Bcl-2 inhibitors have an uncoupling effect on the mitochondria, mitochondrial respiration and ATP synthesis were examined. Mitochondrial respiration was measured by the oxygen consumption at the whole cell level. BH3I-2′ and HA14-1 showed similar effect to CCCP in their ability to stimulate mitochondrial respiration rapidly (Fig. 4*A*). ATP content was evaluated by luciferin-luciferase after the treatment of cells for 4 h. As expected, BH3I-2′, HA14-1, and CCCP also inhibited ATP synthesis (Fig. 4*B*). Treatment with TRAIL did not cause depletion of the intracellular ATP level but in contrast increased the ATP level. With the combination of TRAIL with BH3I-2′, HA14-1, or CCCP, ATP levels were further decreased when compared with those treated with these reagents alone (Fig. 4*B*). This evidence suggests that the cell-permeable Bcl-2 inhibitors have a function in uncoupling mitochondrial respiration from oxidative phosphorylation.

Inhibitors of Bcl-2 but Not CCCP Induce Cytosolic Acidification. One of the functions of Bcl-2 is to maintain the physical H^+ gradient across the MIM, and the proapoptotic protein Bax can cause cytosolic acidification and mitochondrial matrix alkalinization (25). However, CCCP uncouples mitochondrial respiration by dissipating the H^+ gradient across the MIM. We were therefore interested in whether there was a difference between Bcl-2 inhibitors and CCCP in the regulation of cytosolic pH value. Cells were stained with the pH-sensitive dye SNARF-1 acetate, and the change in the cytosolic pH value was measured by flow cytometry. A significant drop in pH value was observed when cells were treated with TRAIL, BH3I-2′, or HA14-1 alone for 4 h. However, CCCP did not cause pH change (Fig. 5). This implies that the uncoupling and the acidification effects of Bcl-2 inhibitors are via a mechanism unrelated to the movement of protons across the MIM.

Uncoupling Effect Enhances TRAIL-Induced Bax Activation and Cytochrome *c* Release. To test whether the uncoupling effect that sensitized TRAIL-induced apoptosis was via the mitochondrial apoptotic pathway, Bax activation and cytochrome *c* release were examined in response to these treatments. Bax conformational change is a key step for the activation of Bax. Cellular proteins were extracted from treated cells and immuno-precipitated with an anti-Bax antibody, clone 6A7, which detects the active form of Bax specifically. TRAIL-induced Bax activation was weakly detected after 4 h of treatment with TRAIL and was greatly enhanced by the cotreatment with BH3I-2′, HA14-1, or CCCP (Fig. 6). The determination of cytochrome *c* release was performed by an immunostaining of triple colors. The mitochondria were stained as a red fluorescence, cytochrome *c* was stained as a green color, and the nuclei were stained with 4′,6-diamidino-2-phenylindole showing blue color (23). Cytochrome *c* in control cells (Fig. 7*A*) or cells treated with BH3I-2′ (Fig. 7*C*) HA14-1 (Fig. 7*E*) or CCCP (Fig. 7*G*) alone localized to mitochondria as red and green-merged pixels appeared orange/yellow. TRAIL-induced cytochrome *c* release was weakly shown in the CEM cells as the green-labeled cytochrome *c* image separating from the mitochondria with a clearly diffuse pattern (Fig. 7*B*), and it was enhanced by cotreatment with BH3I-2′ (Fig. 7*D*), HA14-1 (Fig. 7*F*), or CCCP (Fig. 7*H*).

Caspases Are Not Crucial for TRAIL-Induced Killing When Mitochondrial Respiration Is Uncoupled. Depletion of ATP may affect the apoptotic cell death because ATP is required for cytochrome *c*-dependent caspase activation (26). We therefore tested whether caspase-3 activation was affected when the ATP level was low. Caspase-3 activity was measured by the fluorogenic assay using Z-DEVD-AFC as a substrate. Treatment with TRAIL alone for 4 h increased the activity of the caspase-3. BH3I-2′, HA14-1, or CCCP-treated cells did not show increased caspase-3 activity. However, when the TRAIL treatment was combined with BH3I-2′, HA14-1, or CCCP, all of these uncoupling agents showed a significant synergistic effect on TRAIL-induced activation of caspase-3 (Fig. 8*A*) regardless of the lower levels of ATP. This suggests that the synergistic effect of uncoupling agents on TRAIL-induced apoptosis is caspase dependent.

To evaluate the uncoupling effect on TRAIL-induced killing, a pan-caspase inhibitor, Z-VAD.fmk, was used to inhibit the activation of caspases. Z-VAD.fmk completely blocked the apoptotic cell death induced by TRAIL alone and cotreated with BH3I-2′, HA14-1, or CCCP after 4 h of treatment (data not shown). However, after 6 h of

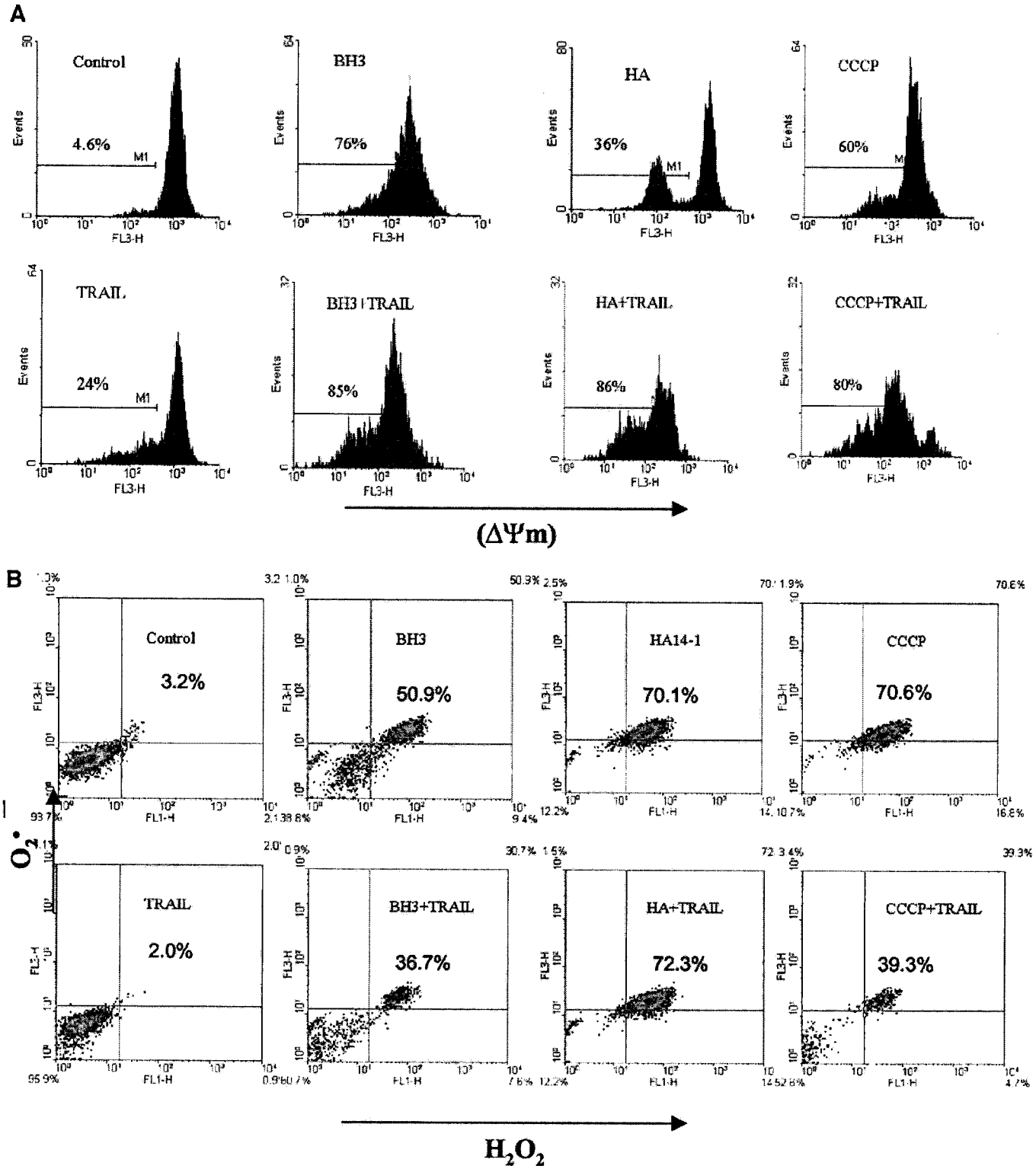


Fig. 2. Effects of BH3I-2′, HA14-1, and CCCP on ΔΨm and ROS production. *A*, ΔΨm. After the treatment, CEM cells were stained with tetramethylrhodamine methylester and analyzed with a flow cytometer at FL3-H channel. Histograms show fluorescence intensity (*X* axis) *versus* cell number (*Y* axis). Cells were gated as M1 are cells with lower ΔΨm ($\Delta\Psi m^{LOW}$). *Numbers* indicated in each graph are percentage of $\Delta\Psi m^{LOW}$ cells. *B*, ROS production. Cells were counterstained with dihydroethidium and 2′,7′-dichlorodihydrofluorescein diacetate. $O_2^{\bullet-}$ generation was represented by increased red fluorescence in the FL3-H channel, and H_2O_2 was measured in the FL1-H channel. The *density dots* in the *top right quarters* are cells that produced both $O_2^{\bullet-}$ and H_2O_2. *Numbers* indicated in each graph are percentage of cells producing ROS. Data presented show representative results of one of three independently performed experiments. *C*, CCCP and TRAIL induced apoptosis. Cells were collected for DNA analysis at 4 and 24 h, respectively. DNA content was analyzed by flow cytometry. Significantly increased sensitivity ($*P < 0.0001$, *t* test) of cells treated with both TRAIL and CCCP (C+T) was statistically compared with cells treated with TRAIL alone. *ΔΨm*, dissipation of the mitochondrial membrane potential; *CCCP*, carbonyl cyanide *m*-chlorophenylhydrazone; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand; *ROS*, reactive oxygen species.

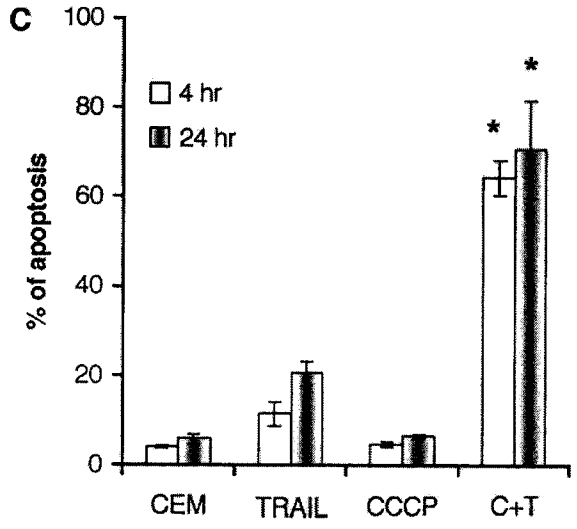


Fig. 2. Continued.

treatment, cells were stained with 10 μg/ml PI, and the integrity of cell membrane was assessed by PI dye exclusion with flow cytometry. The cell membrane of early apoptotic cells remained intact and impermeable to PI dye. Control cells, cells treated with TRAIL and Z-VAD.fmk alone (Fig. 8*B, a-c*), or TRAIL combined with BH3I-2′, HA14-1, or CCCP (Fig. 8*B, d-f*), kept impermeable to PI, indicating the integrity of the cell membrane. However, cotreatment of cells with TRAIL and BH3I-2′, HA14-1, or CCCP in the presence of Z-VAD.fmk for 6 h, part of cells were permeable to PI, show the increased PI fluorescence in the FL3-H channel (Fig. 8*B, g-i*). In addition, BH3I-2′, HA14-1, or CCCP alone did not induce necrosis in the presence of Z-VAD.fmk (data not shown). The MTT test confirmed that the inhibition of caspases failed to block the uncoupling effect-sensitized TRAIL-mediated killing (Fig. 8*C*). Z-VAD.fmk could nor inhibit dissipation of $\Delta\Psi m$ or generation of ROS, which was induced by TRAIL when combined with BH3I-2′, HA14-1, or CCCP (data not shown), indicating that they induce permanent damage on the MIM. These results imply that the uncoupling effect facilitates TRAIL-induced apoptosis when caspases are activated and leads to necrosis when caspases are inhibited.

DISCUSSION

In this study, we found that the inhibitors of Bcl-2, BH3I-2′, and HA14-1 can uncouple mitochondrial respiration at concentrations that could not induce cytochrome *c* release. Their function in dissipation of $\Delta\Psi m$, generation of ROS, stimulation in oxygen consumption, and reduction in ATP synthesis were identical to the uncoupler CCCP. These uncoupling functions were associated with a marked synergy with TRAIL-induced apoptosis via the mitochondrial apoptotic pathway. They also induced necrotic cell death when caspases were inhibited. The synergistic effect of both Bcl-2 inhibitors and CCCP on TRAIL-induced apoptosis was detected in TRAIL-resistant leukemic cell lines, such as CEM, HL-60, and U937 but not in TRAIL-sensitive K562 cell line. This is in agreement with our previous study that TRAIL-induced apoptosis in the K562 cell line is via the mitochondria-independent pathway (7). This study showed evidence, for the first time, that the uncoupling effect can overcome resistance of leukemic cells to TRAIL-induced apoptosis.

The cell-permeable small nonpeptide compounds, BH3I-2′ and HA14-1, are inhibitors for Bcl-2/Bcl-XL, or "BH3 mimetics," and function through their ability to occupy the hydrophobic pocket of Bcl-2/Bcl-XL (12, 13). It has been reported that BH3I-2′ induces apoptosis by neither directly inducing Bax oligomerization and mitochondrial insertion nor by mediating pore formation by Bcl-XL (13). BH3I-2′ and the other BH3Is induce apoptosis by inhibiting the heterodimerization of Bcl-2/Bcl-XL and releasing the proapoptotic Bcl-2 family members, which in turn initiate downstream apoptotic events (13). This presumed that "BH3 mimetic"-mediated apoptosis is indirectly achieved through activating Bax; however, Bax does not form channels on its own but interacts with and/or modulates a pre-existing mitochondrial outer membrane channel. Such a channel is termed the "permeability transition pore," which crosses both mitochondrial membranes at contacting sites and transports

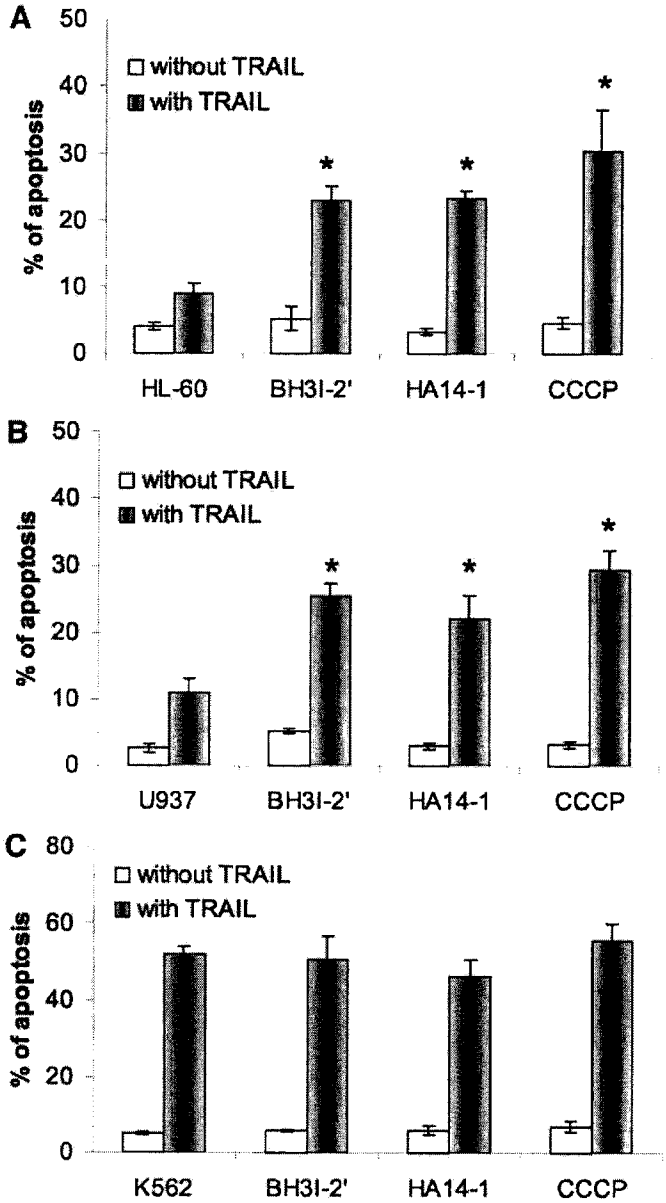


Fig. 3. Synergistic effect of BH3I-2′, HA14-1, and CCCP on TRAIL-induced apoptosis in HL-60, U937 leukemic cell lines, but not in K562 cells. HL-60 (*A*), U937 (*B*), or K562 cells (*C*) were preincubated with or without 30 μM BH3I-2′, 10 μM HA14-1, or 10 μM CCCP for 1 h and then treated with 500 ng/ml TRAIL for 4 h. Apoptosis was measured by flow cytometry as described in "Materials and Methods." Significantly increased sensitivity ($*P < 0.001$, *t* test) was statistically compared in cells that were treated with TRAIL and BH3I-2′, HA14-1, or CCCP with those treated with TRAIL alone. Data shown were mean ± SD from three independent experiments. *CCCP*, carbonyl cyanide *m*-chlorophenylhydrazone; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand.

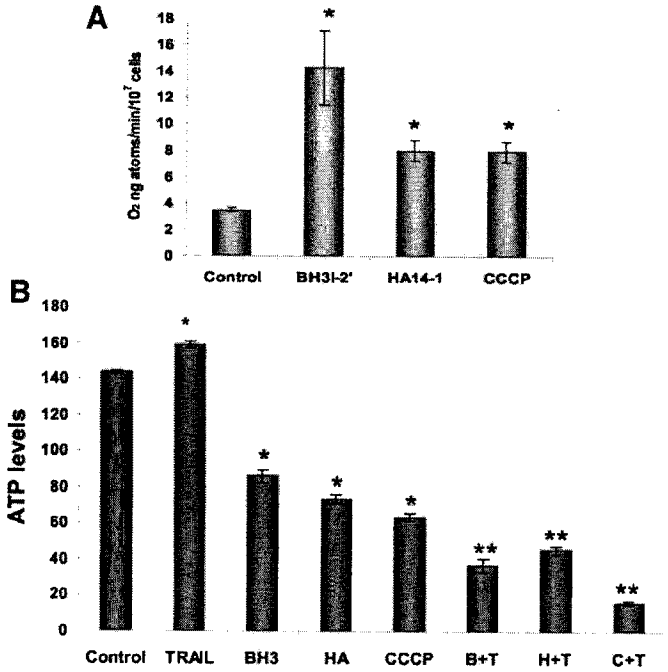


Fig. 4. Uncoupling effect of BH3I-2′, HA14-1, and CCCP. *A*, oxygen consumption. Oxygen consumption was measured by an oxygen electrode. CEM cells (10^7cell/ml) were used for each assay. The respiratory rate was monitored for 3 min. After 30 μM BH3I-2′, 10 μM HA14-1, or 10 μM CCCP was added, the respiratory rate was recorded for another 3 min. The respiratory rate was expressed as nanogram atoms of O_2 per minute per 10^7 cells. Significantly increased respiratory rate after the addition of uncoupling reagent ($*P < 0.001$, *t* test) was compared with the resting respiration of CEM cells. *B*, ATP content. CEM cells were incubated with BH3I-2′ (BH3) or combined with TRAIL (B+T), HA14-1 (HA), TRAIL (H+T), CCCP, or TRAIL (C+T) for 4 h. Cells (10^4) were used for each assay. ATP units were calculated from an ATP standard curve and expressed as nM/10^7 cells. Data shown are mean ± SD from three independent experiments. Significant differences ($*P < 0.001$) of a treatment with a single reagent were analyzed by *t* test by comparison with the control; those treated with double reagents (plus TRAIL) were compared with data treated with a single treatment ($**P < 0.0001$). *CCCP*, carbonyl cyanide *m*-chlorophenylhydrazone; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand.

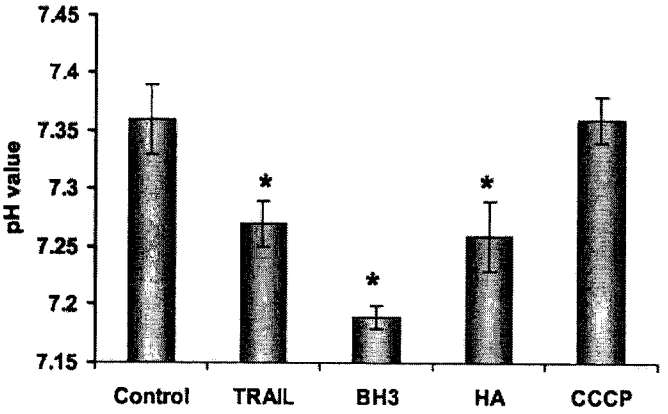


Fig. 5. Effect of the uncouplers on cytosolic pH value. Cells were treated for 3 h and then were stained with SNARF-1. The pH value was calculated from a standard curve that was produced with every single experiment. Data shown are mean ± SD from four independent experiments. Significant decreased pH value in treated cells ($*P < 0.001$, *t* test) was compared with untreated control.

adenine nucleotides and other small molecules (27). Both BH3I-2′ and HA14-1 induced rapid reduction in $\Delta\Psi$m and generation of ROS at concentrations that do not actively induce apoptosis. They have similarities to CCCP on the uncoupling function. In addition to the dissipation of $\Delta\Psi$m and the generation of ROS, they uncouple ATP synthesis from mitochondrial respiration and increase the oxygen consumption. The only difference we observed between the Bcl-2 inhibitors and CCCP is that both BH3I-2′ and HA14-1 induce cytosolic acidification, but CCCP does not. It has been reported that overexpression of Bax induces cytosolic acidification, which can be prevented by Bcl-2, and CCCP does not alter cytosolic pH value (25). This may imply that the uncoupling effect of Bcl-2 inhibitors is not through dissipation of the H^+ gradient across the MIM. The precise mechanism by which Bcl-2 inhibitors uncouple mitochondrial respiration is elusive. Bcl-2 protein has been identified in both MIM (28) and mitochondrial outer membrane (29). Bcl-2 and Bcl-XL are proteins that maintain the integrity of both mitochondrial outer membrane and MIM (30). The cell-permeable property of these Bcl-2 inhibitors may enable them to bind to Bcl-2/Bcl-XL in the MIM and disrupt their role as a gatekeeper of the MIM.

TRAIL did not show the ability to uncouple mitochondrial respiration because it could not inhibit ATP synthesis. The reduction in $\Delta\Psi$m induced by TRAIL was caspase dependent. It has also been reported that neither tumor necrosis factor nor Fas uncouples mitochondrial respiration in the CEM cell line (16, 18). The resistance of CEM cells to death receptor-mediated apoptosis may be largely associated with the lower expression of caspase-8 (18, 24, 31). The requirement for a functional mitochondrial electron transport chain in TRAIL-induced apoptosis has not been widely investigated. Depletion of mitochondrial DNA rendered tumor cells resistant to TRAIL-induced apoptosis (17), and a deficiency in the mitochondrial electron transport chain, which confers resistance to TRAIL, is caused by reduction in both mitochondrial respiration and ATP synthesis. The uncoupling effect, which synergizes TRAIL-induced apoptosis, causes an increase in mitochondrial respiration and a decrease in ATP synthesis. These results imply that mitochondrial ATP synthesis is not crucial for the sensitivity of cells to TRAIL-induced apoptosis. We suggest that increased mitochondrial respiration and ROS generation may be responsible for the synergistic effect of the uncouplers to TRAIL-induced apoptosis. Cytochrome *c*-dependent activation of caspases requires ATP or dATP (26), but the inhibition of ATP synthesis by either Bcl-2 inhibitors or CCCP did not abrogate TRAIL-induced activation of caspase-3. This indicates that the residual levels of intracellular ATP are sufficient for the requirement of cytochrome *c*-induced caspase activation. However, following the inhibition of caspases, TRAIL induced necrosis in the presence of uncoupling agents. This suggests that caspases are not crucial for TRAIL-induced killing in the presence of uncoupler.

Despite the uncoupling effect, both Bcl-2 inhibitors and CCCP at the concentration used for this study did not induce Bax conformational change and cytochrome *c* release. However, they greatly sensitized TRAIL-induced Bax conformational change and cytochrome *c* release. It has been reported that Bax activation is associated with the loss of $\Delta\Psi$m (32) or a rise in intracellular pH value (33). Our results showed that either Bcl-2 inhibitors or CCCP alone did not induce Bax

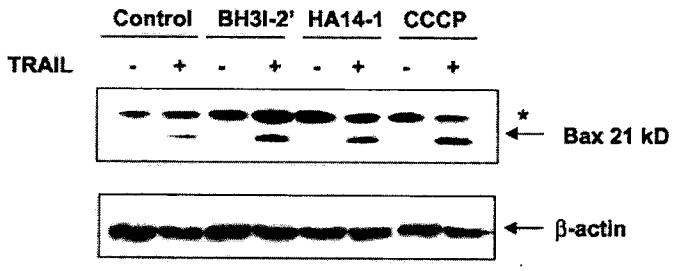
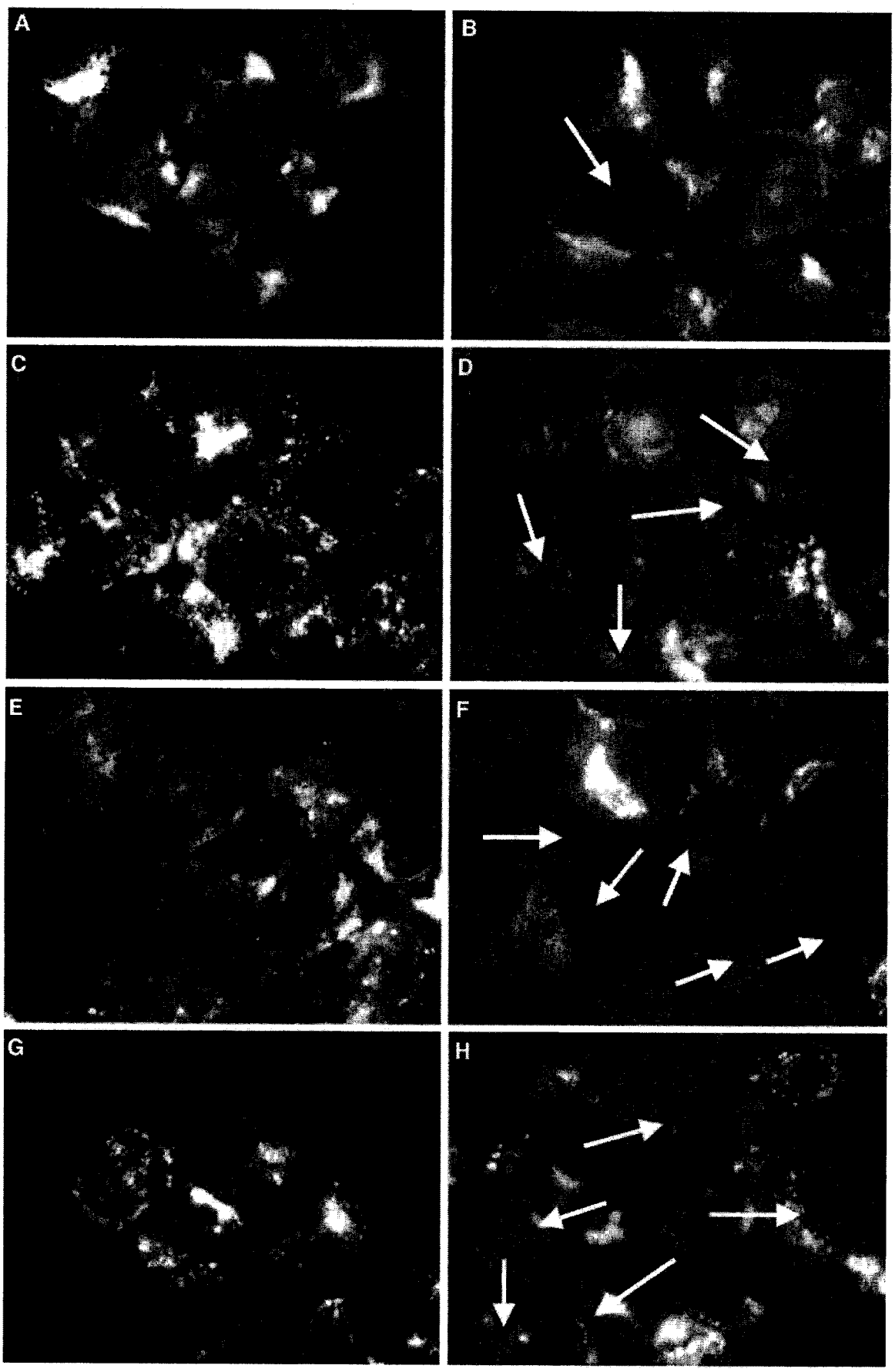


Fig. 6. Detection of Bax conformational change. Cellular proteins were extracted from treated and untreated CEM cells. Proteins (1000 μg) were mixed with Dynabeads that were precoated with the anti-Bax 6A7 antibody and incubated overnight at 4°C. The Western blotting was probed with an anti-Bax antibody 2D2. The conformational changed Bax was detected at 21,000 Daltons. *, IgG light chain. β-actin serves as loading control.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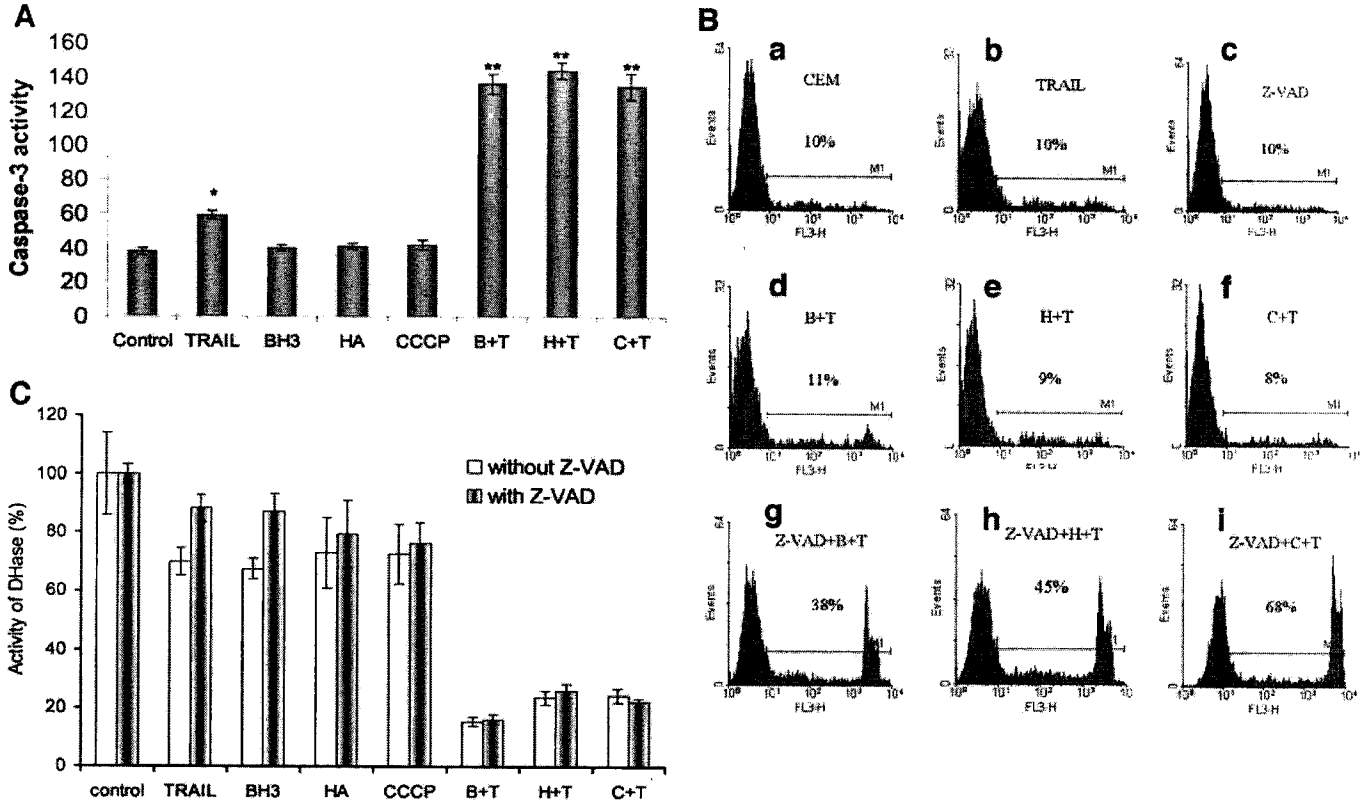

Fig. 8. Effect of caspases in TRAIL-induced cell death. *A*, caspase-activation. Caspase-3 activity was defined as micromolar AFC release per hour per mg protein (μM/h/mg protein). Data shown are mean ± SD from three independent experiments. Significant differences ($*P < 0.001$) of a treatment with a single reagent were analyzed by *t* test when compared with the control. Those treated with combined reagents, such as BH3I-2′ and TRAIL (B+T), HA14-1, and TRAIL (H+T) or CCCP and TRAIL (C+T) were compared with data that were treated with TRAIL alone ($**P < 0.0001$). *B*, necrotic cell death. Control cells (*a*), cells treated with TRAIL (*b*), or Z-VAD.fmk (*c*) alone for 6 h. Cells were pretreated with (*g–i*) or without Z-VAD.fmk (*d–f*) for 1 h and then treated with BH3I-2′ and TRAIL (Z-VAD+B+T), HA14-1 and TRAIL (Z-VAD+H+T), or CCCP and TRAIL (Z-VAD+C+T) for 6 h. Cells were stained with propidium iodide, and the cell membrane integrity was assessed by propidium iodide exclusion with flow cytometry. *C*, MTT test. Cells were treated for 24 h (as indicated for caspase-3 assay) in the presence or absence of Z-VAD.fmk. The cell viability was represented as the percentage of activity of dehydrogenase. Data shown are mean ± SD from three separate experiments. *CCCP*, carbonyl cyanide *m*-chlorophenylhydrazone; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand; *MTT*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

conformational change after the treatment for 4 h. However, both BH3I-2′ and HA14-1 could induce cytochrome *c* release if their concentrations or incubation period were increased. CCCP can trigger Bax translocation in the presence of the F_1-F_0-ATPase oligomycin, suggesting that the ATP level must be maintained for Bax activation, and the collapse in $\Delta\Psi$m is the cause of Bax translocation (32). The uncoupling effect of BH3I-2′, HA14-1, or CCCP decreased the intracellular ATP content, and their ATP-depleting function was further facilitated in the presence of TRAIL. TRAIL-induced Bax activation and cytochrome *c* release were greatly enhanced under the uncoupling condition. An increase in the pH value can induce Bax conformational change *in vivo* and *in vitro* (33); however, the Bax conformational change-induced by TRAIL alone or combined with BH3I-2′/HA14-1 was under acidic conditions. In addition, CCCP that did not change the intracellular pH value also enhanced TRAIL-induced Bax conformational change. Our results suggest that the uncoupling effect on TRAIL-induced Bax conformational change may not be associated with changes in the pH value.

In conclusion, we have shown that Bcl-2 inhibitors, BH3I-2′, and HA14-1 have an uncoupling function on mitochondrial respiration at the concentrations that do not induce cytochrome *c* release. Therefore, similar to the uncoupler CCCP, the uncoupling effect of these reagents greatly sensitized TRAIL-induced Bax conformational change, cytochrome *c* release. Increased mitochondrial respiration and dissipation in $\Delta\Psi$m appears to sensitize CEM cells to TRAIL-induced apoptosis. The uncoupling effect leads TRAIL to induce necrosis when caspases were inhibited. Both Bcl-2 inhibitors and mitochondria uncoupler showed great synergy with TRAIL to overcome leukemic cell resistance to TRAIL-induced killing.

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Fig. 7. Detection of cytochrome *c* release. CEM cells were pretreated with BH3I-2′ (*C* and *D*), HA14-1 (*E* and *F*), or CCCP (*G* and *H*) for 1 h and then incubated without TRAIL (*A, C, E, G*) or with TRAIL (*B, D, F, H*) for 3 h. Cells were then labeled with MitoTracker, fixed, permeabilized, stained with native anti-cytochrome *c* antibody, and finally counterstained with 4′,6-diamidino-2-phenylindole as described in "Materials and Methods." The stained cells were examined by fluorescence microscopy. Cytochrome *c* antibody was visualized with a fluorescent-conjugated antimouse IgG and assigned the color *green*, whereas mitochondria labeled with MitoTracker were assigned the color *red*. Nuclei were stained with 4′,6-diamidino-2-phenylindole and showed *blue* color. Cells that incubated with single agent show punctuated *orange/yellow* mitochondria (when *red* and *green* images were merged). *Arrows* show released cytochrome *c* (*green*), which was separate from mitochondria location (*red*). *CCCP*, carbonyl cyanide *m*-chlorophenylhydrazone; *TRAIL*, tumor necrosis factor-related apoptosis-inducing ligand.

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